N-methyl pyrrolidone/bone morphogenetic protein-2 double delivery with in situ forming implants

Karfeld-Sulzer, Lindsay S ; Ghayor, Chafik ; Siegenthaler, Barbara ; de Wild, Michael ; Leroux, Jean-Christophe ; Weber, Franz E

Abstract: Bone morphogenetic proteins (BMPs) are growth and differentiation factors involved during development in morphogenesis, organogenesis and later mainly in regeneration processes, in particular in bone where they are responsible for osteoinduction. For more than a decade, recombinant human (rh)BMP-2 has been used in the clinic for lumbar spinal fusion at non-physiological high dosages that appear to be causative for side effects, like male sterility. A possible strategy to reduce the effective amount of rhBMP-2 in the clinic is the co-delivery with an enhancer of BMPs' activity. In an earlier study, we showed that N-methylpyrrolidone (NMP) enhances BMP activity in vitro and in vivo. Here we report on the development of a slow and sustained double delivery of rhBMP-2 and NMP via an in situ forming implant based on poly(lactide-co-glycolide). The results showed that the release of NMP can be adjusted by varying the lactide/glycolide ratio and the polymer's molecular weight. The same applied to rhBMP-2, with release rates that could be sustained from two to three weeks. In the in vivo model of a critical size defect in the calvarial bone of rabbits, the implant containing 50 mol% lactide performed better than the one having 75 mol% lactide in terms of defect bridging and extent of bony regenerated area. In situ forming implants for the double delivery of the BMP enhancer NMP and rhBMP-2 appear to be promising delivery systems in bone regeneration.

DOI: https://doi.org/10.1016/j.jconrel.2015.02.019

N-methyl Pyrrolidone/Bone Morphogenetic Protein-2 Double Delivery with In Situ Forming Implants

Authors: Lindsay S. Karfeld-Sulzer[a], Chafik Ghayor[a], Barbara Siegenthaler[a,b], Michael de Wild[c], Jean-Christophe Leroux[d], Franz E. Weber[a,b,e]

[a]University Hospital, Division of Cranio-Maxillofacial and Oral Surgery and University of Zurich, Center for Dental Medicine, Oral Biotechnology & Bioengineering, Frauenklinikstrasse 24, 8091 Zürich.

[b]Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Switzerland

[c]University of Applied Sciences Northwestern Switzerland, School of Life Sciences, Institute for Medical and Analytical Technologies, Muttenz, Switzerland

[d]Institute of Pharmaceutical Sciences, Department of Chemistry and Applied Biosciences, ETH Zurich, Zurich, Switzerland

[e]CABMM, Center for Applied Biotechnology and Molecular Medicine, University of Zurich, Zurich, Switzerland

Correspondence to: Franz E. Weber, University Hospital, Division of Cranio-Maxillofacial and Oral Surgery, Oral Biotechnology & Bioengineering, Frauenklinikstrasse 24, 8091 Zürich. Tel: +41 44 255 5055, Fax: +41 44 255 4179, E-mail: franz.weber@zzm.uzh.ch

Keywords: N-methylpyrrolidone, Bone morphogenetic proteins, poly(lactide-*co*-glycolide), bone regeneration, lactide, glycolide, double delivery

ABSTRACT

Bone morphogenetic proteins (BMPs) are growth and differentiation factors involved during development in morphogenesis, organogenesis and later mainly in regeneration processes, in particular in bone where they are responsible for osteoinduction. For more than a decade, recombinant human (rh)BMP-2 has been used in the clinic for lumbar spinal fusion at non-physiological high dosages that appear to be causative for side effects, like male sterility. A possible strategy to reduce the effective amount of rhBMP-2 in the clinic is the co-delivery with an enhancer of BMPs' activity. In an earlier study, we showed that N-methylpyrrolidone (NMP) enhances BMP activity *in vitro* and *in vivo*. Here we report on the development of a slow and sustained double delivery of rhBMP-2 and NMP *via* an *in situ* forming implant based on poly(lactide-*co*-glycolide). The results showed that the release of NMP can be adjusted by varying the lactide/glycolide ratio and the polymer's molecular weight. The same applied to rhBMP-2, with release rates that could be sustained from two to three weeks. In the *in vivo* model of a critical size defect in the calvarial bone of rabbits, the implant containing 50 mol% lactide performed better than the one having 75 mol% lactide in terms of defect bridging and extent of bony regenerated area. *In situ* forming implants for the double delivery of the BMP enhancer NMP and rhBMP-2 appear to be promising delivery systems in bone regeneration.

1. Introduction

Bone morphogenetic proteins (BMPs), members of the transforming growth factor-β superfamily of proteins, play an extremely important role in bone development and regeneration. The BMP-2 growth factor has been shown to maintain the osteoprogenitor cells, promote differentiation of osteoblasts, and is critical in bone healing [1]. Because of its major role in these processes, there have been significant efforts to deliver BMPs in an efficient manner to regenerate bone and commercial, FDA approved rhBMP products are available, *e.g.* OP-1™ (BMP-7, Olympus, Stryker) or BMP-2 (Infuse Bone Graft, Medtronic, Minneapolis). Numerous studies have explored synthetic polymers, naturally-derived materials, and calcium phosphates in formats including gels, injectable liquids, scaffolds, and particles [2-6]. Despite this body of work, high concentrations of rhBMPs are still needed for effective therapies. Doses on the order of milligrams are delivered to the patient, while physiologic concentrations are several orders of magnitude lower [7]. Various side effects, including ectopic bone formation, urogenital events, male sterility, osteolysis, and neck and throat swelling, can be caused or exacerbated by these high concentrations [8]. Additionally, since BMPs are produced recombinantly, they are very expensive treatments, especially when large doses are required.

Previously, our laboratory discovered the enhancing effects of *N*-methyl pyrrolidone (NMP) on rhBMP-2, which offers a means to lower the efficacious delivered BMP dose [9]. NMP has been demonstrated to both increase bone forming activities of osteoblasts and decrease bone resorption of osteoclasts [10]. An *in vivo* study using a rabbit calvarial defect model showed significantly higher bone regeneration with NMP

alone by increasing the activity of the natural locally deposited autologous BMP [9]. Furthermore, NMP is used as an injectable pharmaceutical excipient in FDA approved formulations and regarded as safe up to certain concentrations [11, 12]. Moreover, NMP is inexpensive and easily attainable; it is commonly used in various industries as a plasticizer and solvent.

To be able to take full advantage of the bone augmenting properties of NMP, it is important to retain NMP and deliver it over a sustained period of time. Previous studies have demonstrated the importance of slow delivery of rhBMP to obtain the best possible bone regeneration and provide the safest therapy [2, 13]. However, NMP, which is a small water and organic miscible molecule, is not easy to deliver slowly; NMP is cleared from the body within a few hours [14, 15]. Typically, conventional drug delivery strategies are not adapted to this type of molecule.

One drug delivery system that was developed to deliver a variety of different compounds utilizes NMP or another organic solvent to dissolve polyesters along with the drug substance. Upon immersion in an aqueous solution, a phase separation occurs in this so-called *in situ* forming implant (ISFI), and the water insoluble polymer precipitates to form a membrane surrounding a liquid interior [16]. Through diffusion of water into the ISFI and the efflux of the organic solvent, the drug is released over a sustained period of time with the release profile dependent on multiple factors, including the solvent, polymer properties, and composition. Four products based on Atrigel®, an ISFI system developed by Atrix Laboratories, were FDA approved. These four products are (1) Atrisorb® without any drug for periodontal regeneration, (2) Atridox® with doxycycline for subgingival delivery for periodontal treatment, (3) Doxyrobe® with doxycycline for animal

periodontal treatment, and (4) Eligard™ with leuprolide acetate for prostate cancer therapy [17]. Several of the clinical applications of ISFIs target bone, with Atridox® and Atrisorb® for alveolar ridge augmentation and other periodontal bone defects [17-19], and an ISFI combined with tricalcium phosphate for non-weight bearing orthopedic applications [20].The ISFI system possesses many benefits, including injectability, fast application, space filling without the need to cut material to size, and biocompatibility. Furthermore it was shown to be bioadhesive to bone and teeth and to facilitate local delivery and limited pain [18, 21].

Instead of using the ISFI system to deliver a classical drug, we have explored this technology to extend the release of NMP, which itself is a bioactive substance [9, 10]. By examining the dependence of the release profile on polymer properties, we have prolonged the release of NMP from hours to a few weeks. Moreover, this system has been expanded to also include BMP-2 for a greater enhancement of bone regeneration, rather than relying solely on the amount of growth factor that is naturally present. A calvarial defect model study in rabbits was performed to demonstrate the efficacy of this double delivery system.

2. Materials and Methods

All animal procedures were approved by the local authorities (Kantonales Veterinäramt, Zürich, Switzerland, approval 107/2012) and are in line with the European Committee Council Directive Ref. 86/609/EEC.

2.1 Implant Formation

Poly(DL-lactide) (PLA) and poly(DL-lactide-*co*-glycolide) PLGA were obtained from Boehringer Ingelheim (Ingelheim am Rhein, Germany). Polymers were dissolved overnight-shaking at 37°C to final 40 wt% in pure NMP (Sigma-Aldrich, St. Louis, MO) or a combination of NMP with water. Table 1 lists the polymers, with the weight average molecular weight and polydispersity as measured with gel permeation chromatography (see below for methods) and percentage lactide and glycolide

Table 1: List of polymers used in the ISFIs

Polymer Name	Weight average molecular weight (M_w) [kDa]	Polydispersity index	Lactide [mol%]	Glycolide [mol%]
RG502	14.5	2.06	50	50
RG503	42.6	2.25	50	50
RG752	12.2	1.99	75	25
R202	9.7	2.04	100	0

2.2 NMP Release

Release studies were performed by injecting 500 mg of the dissolved polymer into a cylindrical stainless steel mesh basket (10 mm diameter; 13 mm height; 0.6 mm mesh size) and immediately immersing it into 300 mL of phosphate buffered saline (PBS), 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 KH_2PO_4, pH 7.4. With the mesh suspended in the middle of the liquid, samples were maintained at 37°C and shaking at 100 rpm. At predefined time points (30 minutes, 1, 2, 4, 6, 8, and 24 hours, 2, 3, 7, 10, 14, 21, and 28 days), a 1-mL aliquot of release buffer was removed and saved

for later analysis. NMP concentration was analyzed using liquid chromatography-mass spectrometry on a Thermo Scientific LTQ XL instrument (Thermo Fisher Scientific, Waltham, MA) with a Hypersil Gold column (100 x 1 mm dimensions, 1.9 µm particle size, Thermo Fisher Scientific). A gradient of water and methanol, each with 0.1% formic acid was used to analyse the samples, producing a UV peak that corresponded to the molecular weight of NMP. NMP concentration was quantified using the area under the curve of the peak of the UV signal at 210 nm compared to established NMP standards with a limit of quantification of 0.0625 mM NMP. There were at least three samples per group.

2.2 Degradation

The degradation study was performed similarly to the release study except that implants were removed at specified time points, blotted to remove excess buffer, and frozen at -80°C until analysis. Initial polymer molecular weights and polymer degradation samples were analyzed with gel permeation chromatography with refractive index detection and polyethylene glycol standards for molecular weight calibration. Samples and standards were dissolved in dimethylformamide with 0.01 M LiBr and run at 45°C on a Viscotek GPCmax instrument with OmniSEC software (Version 4.6.0) (Malvern Instruments Ltd, England, Enigma Business Park, Grovewood Road, Malvern, Worcestershire, UK).

2.3 BMP-2 in Vitro Studies

Bone morphogenetic protein-2 produced in *E. coli* in our laboratory (detailed expression and purification procedures can be found in prior literature [22]) was used for initial studies analyzing activity in different conditions and for release experiments. To test the activity of rhBMP-2 in various processing conditions, rhBMP-2 dissolved in acetic acid was lyophilized and resuspended in either acetic acid or NMP. Subsequently, it was further diluted in acetic acid, NMP, or combinations of the two. The activity of rhBMP-2 was measured by incubating samples with C2C12 cells and measuring alkaline phosphatase as early marker for bone formation. C2C12 cells (American Type Culture Collection, Manassas, VA) were cultured with DMEM (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (Life Technologies), 1% 200 mM L-glutamine (Life Technologies) and 1% penicillin-streptomycin (10000 units/mL pen, 10000 µg/mL strep, Life Technologies). Cells were maintained at 37°C in a humidified chamber with 5% CO_2. After 5-7 days incubation time, media was removed; cells were washed in Dulbecco's PBS (Life Technologies), and incubated at room temperature for 30 minutes with 0.56 M 2-amino-2-methyl-1-propanol (Sigma-Aldrich). Subsequently, cells were scraped from the plate and homogenized for 10 s. The cell lysate supernatant was combined with an equal volume of 0.56 M 2-amino-2-methyl-1-propanol (Sigma-Aldrich) with 4mM $MgCl_2$ and 20 mM disodium p-nitrophenyl phosphate (Sigma-Aldrich). Samples were heated at 37 °C for 10 min, NaOH was added to quench the reaction, and the optical density was read at 410 nm to measure alkaline phosphatase activity.

After initial testing of different conditions, the rhBMP-2 dissolved at 1 mg/mL in 1 mM HCl was added to the polymer and NMP by shaking at 37°C for 4-6 h for the release

studies. As in the NMP release studies, polymers were 40 wt% in NMP plus BMP solution. The dissolved polymer with 10 µg rhBMP-2 was injected into a metal mesh basket and placed in 2 mL PBS with 6 M urea and maintained at 37°C on a turning-wheel at 100 rpm. Additionally, rhBMP-2 at the same concentration in the PBS/urea buffer were used as controls. Individual samples and controls were removed at each timepoint (7, 14, 21, and 28 days) and the supernatant was decanted and frozen at -20°C until analysis.

A Bio-Dot SF Microfiltration Apparatus (Bio-Rad, Hercules, CA) containing 48 slots with a nitrocellulose membrane was used to measure the concentration of released rhBMP-2 as described earlier [23]. 200 µL of standards and appropriately diluted samples were added to the wells. The membrane was blocked for 1 h with NET-gelatin, incubated for 1 h with an anti-BMP-2/4 primary antibody (R&D Systems, Minneapolis, MN) diluted 1:500 and for 1 h with a polyclonal rabbit anti-goat IgG/HRP secondary antibody (Dako, Glostrup, Denmark) diluted 1:2000. After Electrochemiluminescence detection procedures, the film was scanned and analyzed with ImageJ (Version 1.47h, Wayne Rasband (NIH), rsb.info.nih.gov/ij/) to compare intensities of the test and control samples and standards. Samples are normalized to the rhBMP-2 controls to account for growth factor degradation.

2.4 Rabbit Implantation Studies

24 adult (12 month old) New Zealand white rabbits weighing between 3.5 and 4.2 kg were used in this study with six rabbits per group. A 15 mm circular full-thickness

defect was created in the center of the calvaria using an electric burr. The bone was carefully removed, ensuring not to disturb the dura. A stepped titanium scaffold with an upper diameter of 17 mm, a lower diameter of 15 mm, and a total height of 3.8 mm was placed in the defect site. The titanium scaffold was produced by additive manufacturing using selective laser melting as described previously [24]. Rod thickness was 0.2 mm. After fabrication, all samples were ultrasonically cleaned in a 4% Deconex© 15PF (Beiersdorf, Münchenstein, Switzerland) solution at 90°C for 5 min, then cleaned thrice in ultrapure water (resistivity 18.2MΩcm) for 15 min in an ultrasonic bath, and twice passivated in concentrated nitric acid (65% HNO_3; Fluka) under ultrasonic agitation. The implants were processed in high purity oxygen RF plasma (PDC-32G; Harrick, oxygen purity 99.9995%, Carbagas) at 29.7W for 5 min in order to clean and sterilize the surface. Before the *in vivo* tests, the samples were packed and gamma sterilized with 25 kGy. Rabbits were sacrificed after 16 weeks and the calvaria were removed for histological processing as described [24]. Four different conditions were tested: titanium scaffold alone, titanium scaffold with RG502, titanium scaffold with RG502 containing 12 µg rhBMP-2 and titanium scaffold with RG752 containing 12 µg rhBMP-2. For the groups containing polymer, 200 µL of 40 wt% RG502 or RG752 dissolved in NMP with or without BMP-2 was deposited onto the center of the scaffold immediately before implanting.

2.5 Histology

The samples were first prepared with a sequential water substitution by ethanol process. Defatting took place in xylene. Next, the samples where infiltrated with methyl

methacrylate (MMA, Aldrich Chemistry, Japan) and embedded by adding to 100 mL MMA, 3 g of di-benzoylperoxid and 10 mL plastoid N (Merck AG, Zug, Switzerland). Polymerization was allowed to occur at 37°C in an incubator. Sections were prepared from the midst of the defects and ground to 25–50 µm. Thin-ground sections were stained with Coomassie brilliant blue and digital images taken to determine the bony regenerated area and the bony bridging via pixel measurement using ImageJ (Version 1.47h, Wayne Rasband (NIH), rsb.info.nih.gov/ij/).

Bony regenerated area is defined as the fraction of the titanium scaffold where bone formation has occurred. It includes bone, bone marrow and the scaffold embedded in bone. Bone bridging was determined in the middle section. First, the areas with bone tissue were projected onto the x-axis. Next, the stretches of the x-axis where bone formation had occurred at any level were summed up as described earlier [25, 26]. Bone bridging is given in percentage of the defect width (15 mm) where bone formation has occurred.

2.6 Statistics

The primary analysis unit was the animal. For all parameters tested, treatment modalities were compared with a Kruskal-Wallis Test, followed by Mann-Whitney signed rank test for independent data (IBM SPSS v.19). Significance was set at $P < 0.05$. Data from six rabbits per group are presented. Values are reported as mean ± standard deviation and displayed as box-plots ranging from the 25th (lower quartile) to the 75th

(upper quartile) percentile including the median and whiskers showing the minimum and maximum values.

3. Results

3.1 NMP Release

Various polymers were tested to determine the effects of different factors on the release of NMP from the *in situ* forming implants. PLGA with different molecular weights but the same monomer ratio of 50% lactide and 50% glycolide were studied. The implants reproducibly filled the diameter of the mesh basket with a similar globular shape and a maximum 6% weight variation amongst implants. As shown in Figure 1a, higher polymer molecular weight led to faster release in the initial 24 h. RG502 released only 14.5% in the first 24 h, a low burst amount, whereas RG503 lost significantly more NMP, over 50% during this time period. However, the release rate of RG503 decreased after the initial 24 h and RG502's rate increased after a brief stagnation, causing the release from RG502 to surpass that of RG503 after 10 days (Figure 1b).

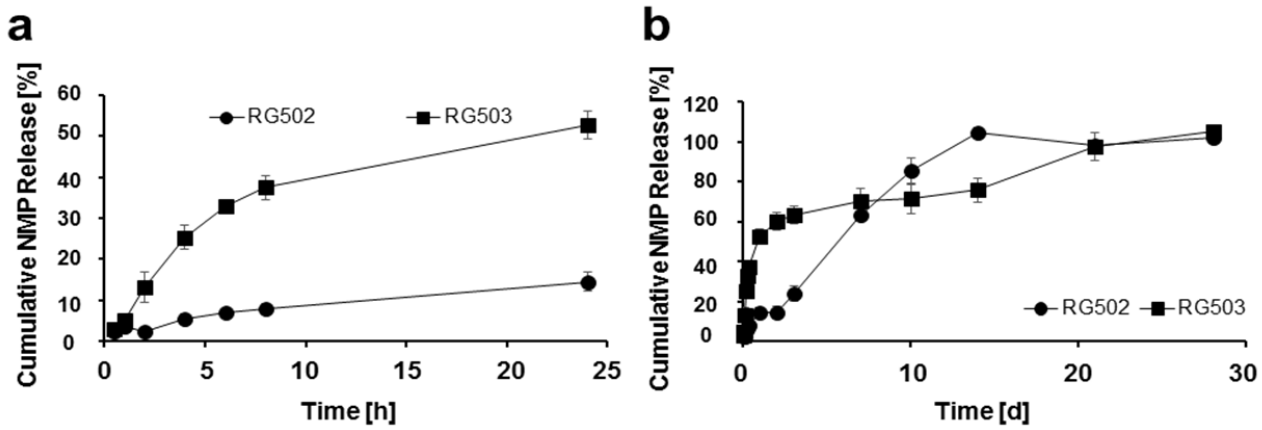

Figure 1: NMP release dependence on molecular weight. Weight average of RG502 is 14.5 kDa and of RG503 42.6 kDa. Cumulative NMP release of ISFI with 40% PLGA and 50 mol% lactide. Release over (a) 24 h (b) and 28 d. Mean ± SD (n=3).

The crossover point occurred at approximately day 7. At days 7 and 10, the release from the two polymers was not statistically different. By day 14, the RG502 had released 100% of the NMP, whereas RG503 released only about 75%. RG503 then released all of the NMP by day 21.

In addition to comparing molecular weight, the ratio of the lactide and glycolide monomers was examined for its impact on NMP release. Figure 2a demonstrates the impact of varying the lactide percentage using polymers with the lowest molecular weight, with lower lactide percentages leading to less NMP release in the first 24 h. For example, R202, with 100 mol% PLA, released over twice as much of its NMP (40.6%) *versus* RG502 with 50 mol% lactide (14.5%). Over the course of four weeks, the results were reversed with the highest lactide content retaining NMP for the longest period of time, as seen in Figure 2b. RG502 (50 mol% lactide) reached 100% by day 7, and RG752 (75 mol% lactide) had a greater release than R202 (PLA) by day 14. After 28 days, R202 (100% PLA) had only released 75% of the NMP, whereas 50 and 75 mol%-lactide polymers freed all of the NMP earlier, at 14 and 21 days, respectively.

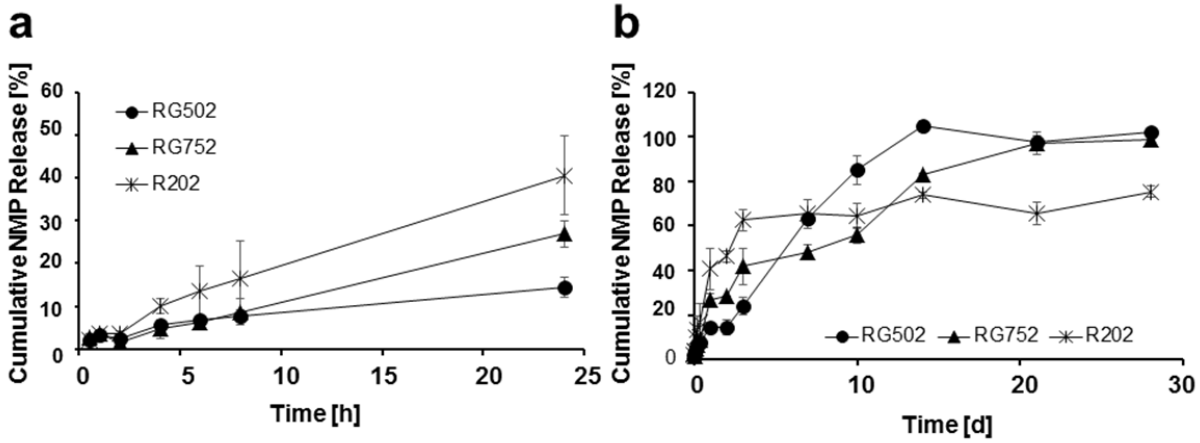

Figure 2: NMP release dependence on lactide percentage. Lactide content for RG502 amounts to 50 mol%, of RG752 to 75 mol% and of R202 to 100 mol%. Cumulative NMP release of ISFI with 40% PLGA and the lowest molecular weight. Release over (a) 24 h (b) and 28 d. Mean ± SD (n=3).

3.2 ISFI Degradation

The degradation profiles of the ISFIs with the two polymers containing 50% lactide, RG502 (14.5 kDa) and RG503 (42.6 kDa), were evaluated. Using GPC analysis, the molecular weight distribution of the ISFI was measured at multiple time points from 1 to 28 days. As shown in Figure 3, the average M_w of RG503 was initially much higher and the degradation rate steeper. However, by day 21, the RG503 ISFI M_w was no longer different from RG502.

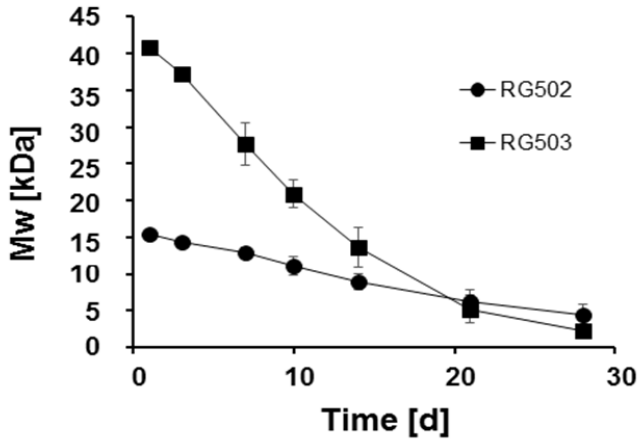

Figure 3: Degradation of ISFI prepared with RG502 or RG503 in PBS. Mean ± SD (n=3).

3.3 BMP-2 Release

In order to include rhBMP-2 in the ISFI system, studies were performed to determine the effects of the NMP ISFI system on the activity on BMP-2 and of the BMP-2 on the NMP release. Since BMP-2 must be folded correctly to retain its activity and NMP could potentially disturb its secondary/tertiary structure, different process methods were evaluated. BMP-2 lyophilized and resuspended in 100% NMP resulted in a complete loss of activity, as measured by an alkaline phosphatase assay.

Furthermore, rhBMP-2 was no longer active when directly diluted in mixtures of NMP and acetic acid, even in 10% acetic acid/90% NMP (w/w), as seen in Figure 4. The fact that the polymers used in the ISFI system are not water soluble in higher than 10% aqueous solutions, limited the water content in the ISFI. Although rhBMP-2 directly dissolved in acetic acid/NMP mixtures inactivated the growth factor, rhBMP-2 dissolved in an aqueous acetic acid solution and then further diluted in NMP was active. Due to system constraints, the maximum percentage of NMP that could be tested was 97.5%, but the rhBMP-2 activity in this condition was equivalent to the controls of rhBMP-2 and NMP separately added to cells in the alkaline phosphatase assay. Therefore, particular processing methods maintained the activity of BMP-2 in a highly organic solvent environment.

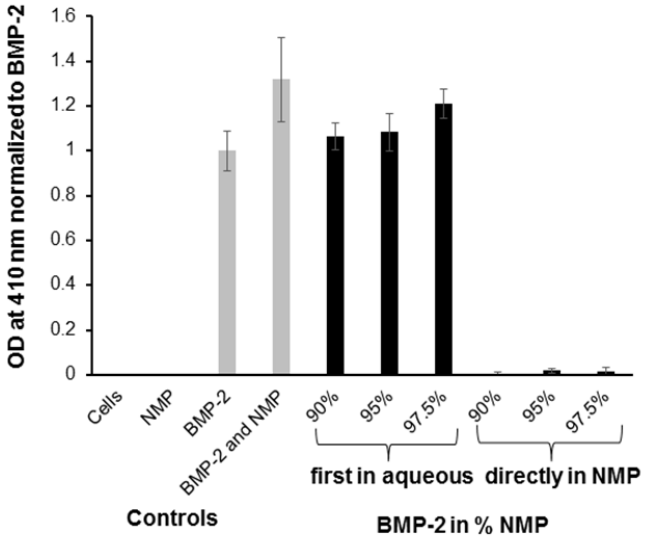

Figure 4: Activity of BMP-2 with NMP. Alkaline phosphatase activity was measured with lyophilized BMP-2 directly diluted in various percentage (w/w) NMP solutions with acetic acid (black bars) or BMP-2 first dissolved in an aqueous acetic acid solution and then diluted in various percentage NMP solutions (gray diagonal hashed bars). Cells only and NMP served as negative controls and BMP-2 (in aqueous solution) and BMP-2 (in aqueous solution) and NMP added directly to the well served as positive controls (solid gray bars). All data is normalized to the positive control of BMP-2 (in aqueous solution). Mean ± SD (n=3).

Since the rhBMP-2 must be partially dissolved in aqueous solution to remain active, the effect of aqueous solution included in the IFSI system on the NMP release was tested using RG502, the lowest molecular weight polymer with 50 mol% lactide. During the first 24 h, the NMP release was slightly lower when water was included, although this difference was not significant (Figure 5). Over the course of 28 days, the cumulative NMP release was entirely equivalent for solvent with only NMP or with up to 10% (w/w) water. It is important to note that the concentration of NMP was different since the total amount of NMP was higher when only NMP was used as the solvent. Thus, the addition of a small amount of water did not affect the ISFI system release characteristics.

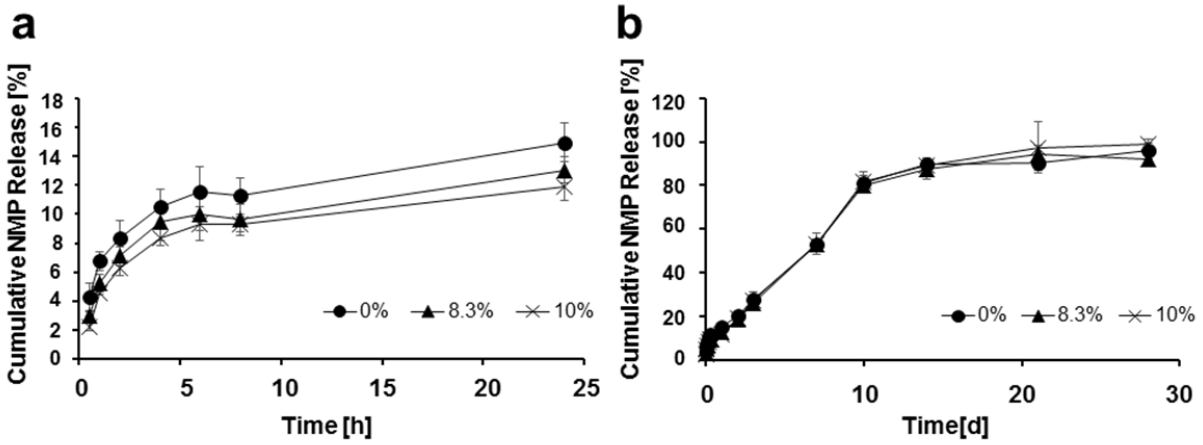

Figure 5: NMP release dependence on percentage of water in the solvent. Cumulative NMP release of ISFI with 40% PLGA, 50 mol% lactide, and the lowest molecular weight (RG502). The solvent dissolving the polymers was entirely NMP (no water), contained 8.3 or 10% (w/w) water. No BMP-2 is included in these formulations. Release over (a) 24 h (b) and 28 days. Mean ± SD (n=3).

The preliminary studies showed that there was potential to include rhBMP-2 without dramatically changing the ISFI system to enable delivery of an active growth factor. Therefore, rhBMP-2 was added to the polymer and NMP in an acid solution as

10% (w/w) of the total solvent. rhBMP-2 release, measured at weekly intervals up to 28 days, indicated that there was a very low level of release initially for both RG502 (50 mol% lactide) and RG752 (75 mol% lactide) (Figure 6). By 21 days, release dramatically increased, with practically all of the rhBMP from the RG502 ISFI ending up in the supernatant whereas about only half of the rhBMP in the RG752 IFSI was released. At 28 days, both polymers had released essentially all of the rhBMP.

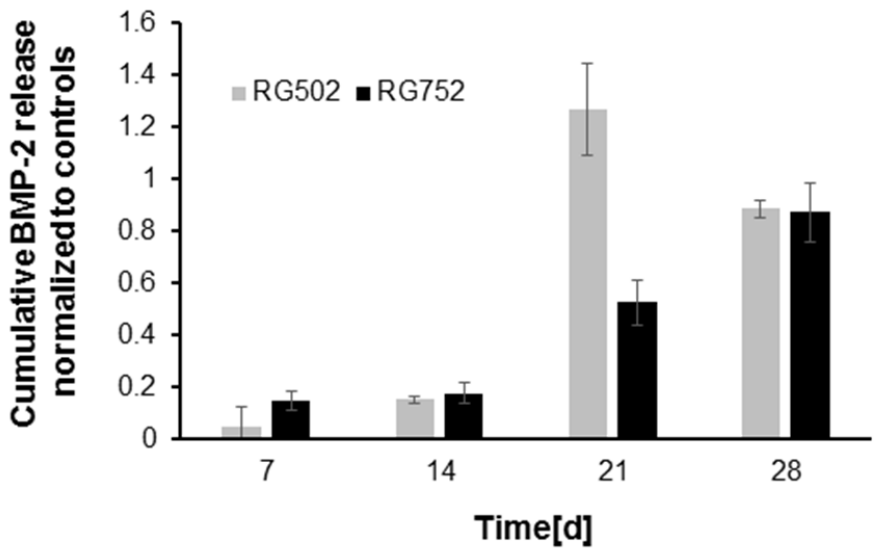

Figure 6: BMP-2 release dependence on lactide percentage. BMP-2 was included in the ISFI solutions and released into a PBS-urea buffer. BMP-2 was measured with a Bio-Dot apparatus with a primary anti-BMP-2/4 antibody. All data are normalized to the control of rhBMP-2 in PBS-urea at the same timepoint. Mean ± SD (n=3).

3.5 In Vivo Bone Formation with ISFI

The ISFI with 50% lactide (RG502) and 75% lactide (RG752) were tested *in vivo* in a cranial defect model in rabbits. Since the degradation characteristics of the materials would also influence the healing of the defect, a titanium based scaffold was

used (Fig. 7a) so that the ISFI could be placed in the center of the defect (Fig. 7b). By doing so, the presence of the ISFI would not directly interfere with bone regeneration occurring predominantly from the defect margins besides the exposure to NMP, rhBMP-2 and possibly degradation products released from the ISFI. For all the animals, the healing over a 16 week time period was uneventful. The histological sections revealed that bone formation has occurred in this critical size defect model to a great extent (Fig. 7e-g). Defect bridging between the four groups was not significantly different (Fig. 7h). Without ISFI, 78 ± 35% of the defect was bridged. With NMP loaded 50% lactide ISFI defect bridging occurred to 49 ± 18%. In the presence of the NMP/rhBMP loaded 75 mol% lactide ISFI, defect bridging occurred to 70 ± 19% and with the NMP/rhBMP loaded 50 mol% lactide ISFI, the mean defect bridging amounted to 83 ± 20%.

Without ISFI, 28 ± 15% of the implant was bony regenerated. With NMP loaded 50% lactide, ISFI bony regeneration occurred to 21 ± 9%. In the presence of the NMP/rhBMP-2 loaded 75 mol% lactide ISFI (RG752), bony regeneration occurred to 29 ± 8%, and with the NMP/rhBMP-2 loaded 50 mol% lactide ISFI (RG502), the mean bony regeneration percentage was 50 ± 12% (Fig. 7k). Bony regeneration with the NMP/rhBMP-2 loaded 50 mol% lactide ISFI (RG502) was significantly better than with NMP/rhBMP-2 loaded 75 mol% lactide ISFI (RG752) (P=0.016), than with NMP loaded 50% lactide (P=0.003), and than without ISFI (P=0.041). Therefore, for this critical size defect treated with a titanium-based scaffold, the rhBMP-2/NMP release profile of the 50 mol% lactide ISFI was significantly better suited to enhance bony regeneration than that of the 75 mol% lactide ISFI.

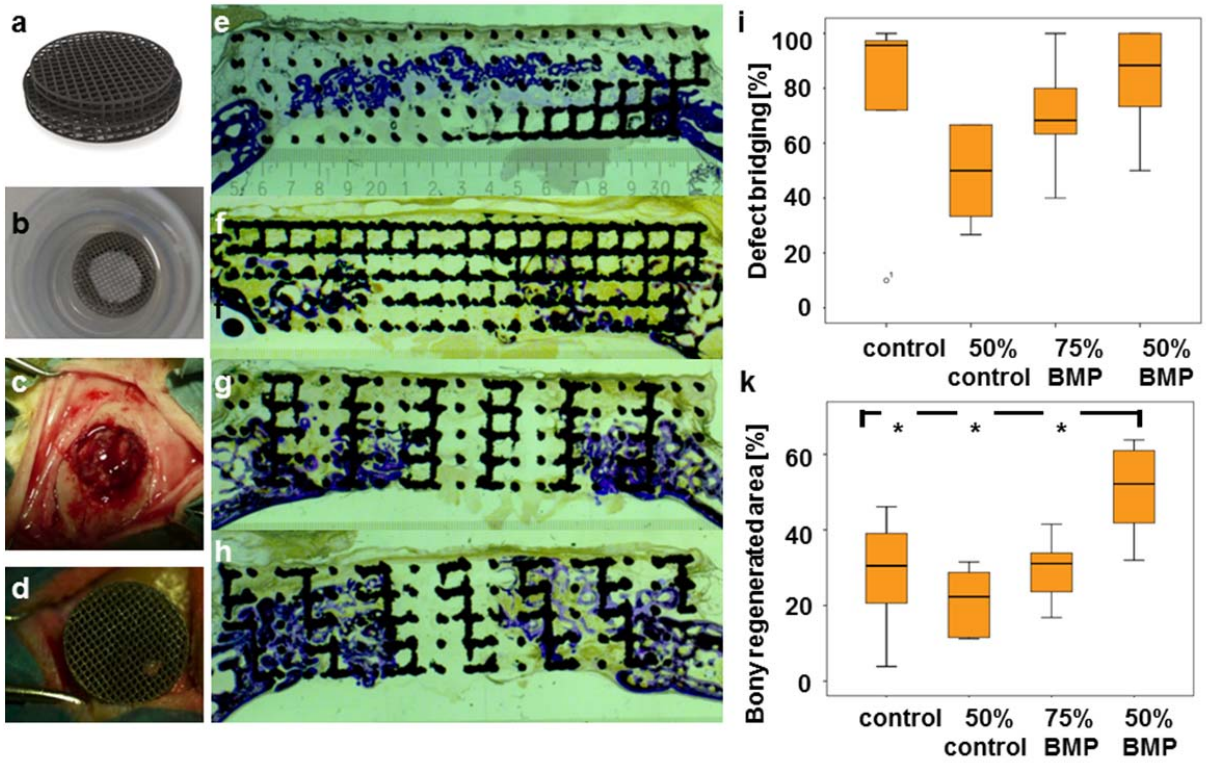

Figure 7: *In vivo* bone formation. Titanium based lattice scaffolds (17/15 mm) in diameter were manufactured by selective laser melting, filled with ISFI and implanted in the calvarial bone of rabbits. a) Design of the scaffold. b) Loading of the scaffold with ISFI. c) Defect before implant placement. d) Placement of scaffold into the bony defect. Histology of the middle section: e) control is scaffold only, f) scaffold with ISFI 50% mol% lactide without BMP, g) scaffold with ISFI 75 mol% lactide with BMP, h) scaffold with ISFI 50 mol% lactide with BMP, scale in mm is provided in e; bone appears blue with Toluidine-blue staining, titanium black. i) Box–plot for defect bridging and k) bony regenerated area. * indicates P values<0.05. Exact P values are provided in the result section. Mean ± SD (n=6).

4. Discussion

Since our laboratory's discovery of NMP's enhancing effects on BMP-2 and bone regeneration, it has been a goal to deliver this molecule in a sustained manner to take full advantage of its benefits. However, since NMP has a very low molecular weight (99.1 g/mol) and is both water and organic-soluble, it is difficult to retain in a delivery system. If the release of NMP could be extended to at least a couple of weeks, the NMP

would be available in the time period when BMP is exerting its action for regenerating bone.

Here we report that the ISFI system with polyesters was successfully engineered to deliver NMP for more than two weeks, depending on the particular polymer used. The total duration of 100% release of NMP varied based on both polymer molecular weight and the ratio of the monomers. Lower molecular weight and higher lactide percentage polymers retained NMP for an overall longer time. These data correspond to other work that indicates that the nature of release profiles in the later time points is due to polymer degradation [27]. The polyesters with higher amounts of lactide exhibit slower degradation rates due to the more hydrophobic characteristic of the lactide monomer with the extra methyl group that hinders water access [28]. Liu and Venkatraman confirmed this by demonstrating that a 75 mol% lactide PLGA ISFI degrades slower than a 50 mol% lactide polymer, and had NMP release profiles that generally agreed with those found here [27]. Solorio et al. confirm similar trends in molecular weight degradation for ISFI with different initial molecular weights [29].

In addition to extending the release of NMP to a sufficiently long time frame, ISFI formulations were found that also restricted burst to a low amount in the first 24 h by using low molecular weight and low lactide percentage polymers. The pattern of burst followed by slow release has been previously shown to be typical for ISFI systems with PLGA and NMP [30]. However, high burst levels of NMP could potentially lead to toxic elevated concentrations of NMP. The amount of NMP burst increased with higher molecular weight, which corresponds to other studies that show greater burst release of small molecule drugs with higher molecular weight polymers [31, 32]. Prior research has

indicated that ISFIs that undergo fast phase separation result in a more porous morphology that accelerates initial release [16, 30, 33, 34]. A major contributing factor to the rate of phase inversion is the hydrophobicity of the polymer with a lower water affinity leading to more rapid phase transitions. Higher molecular weight polymers have been demonstrated to have lower water solubility, indicating that they are more hydrophobic [31], which can explain the relationship between burst and molecular weight demonstrated here. The higher burst rate of polymers with higher lactide content, which agrees with literature [27], can also be attributed to hydrophobicity. Since lactide is more hydrophobic than glycolide [28], the higher amount of lactide will lead to polymers of lower hydrophilicity and a faster transition rate.

In order to create a more robust bone regeneration system, exogenous rhBMP-2 was integrated into the ISFI to supplement the natural BMP-2 to enable greater NMP enhancement of BMP-2's osteogenic effects for improved bone regeneration. By introducing rhBMP-2 already dissolved in an aqueous solution into NMP, the rhBMP-2 retained its activity. Analysis of the additional water in the system mandated by the inclusion of rhBMP-2 in an aqueous solution had no significant impact on the NMP release rates even though polymer solubility differences between water and the solvent are the fundamental principle of formation of ISFIs. Since PLGA remains soluble with only a small percentage of water (6-12% aqueous solution for similar polymers in NMP) [20], only low levels of water could be included.

rhBMP-2 release from the ISFI was studied using the two most promising polymer candidates that combine a low burst with long term NMP release: the lowest molecular weight polymers with 50 and 75 mol% lactide (RG502 and RG752). rhBMP-2 release

followed trends of the NMP release, although lower relative levels of BMP-2 release are seen until day 21. Overall, the ISFI retained the rhBMP-2 for an extended period of time with only a small amount released in the first two weeks.

Previous animal studies with ISFIs and rhBMP by others are limited to a pilot study in rats [19, 35], that indicated that the system is osteoinductive and healed periodontal defects better than a collagen delivery vehicle. An optimization in terms of NMP and rhBMP release for bone regeneration has not been performed so far. Here bone regeneration was analyzed by two ISFI systems containing the same amount of rhBMP-2 and NMP, however, releasing them with different kinetics. For the application of the ISFI we used titanium scaffolds (Fig. 7a) that can be permanently implanted and have inherent osteoconductive properties, as demonstrated in a rabbit calvarial defect model [24]. However, the primary role of the scaffold in this study is to serve as a carrier. Since the ISFI was placed in the middle of the scaffold a physical interference with bone in-growth occurring from the defect margins was omitted (Fig. 7b).

The results revealed that compared to the titanium scaffold without ISFI and the titanium scaffold with the ISFI system with 50% lactide without rhBMP, only the ISFI system with a 50% lactide containing polymer performed significantly better. The same amount of NMP and rhBMP-2 released with different kinetics from the 75 mol% lactide containing polymer had no positive effect on bone regeneration determined 112 days post-surgery. A possible direct effect of the slower degradation of the 75% lactide compared to the 50% lactide was minimized in our system, since the ISFIs were placed in the center of the titanium scaffold. From the literature it is known that 80% degradation of the 75 mol% lactide implant is reached at 48 days compared to 30 days

for the 50 mol% lactide system [27]. The healing model used here continued until day 112 post surgery, leaving sufficient time for undisturbed healing to occur for both materials.

NMP and BMP release profiles, not degradation, are suspected to be the major contributors to differences in bone regeneration between the RG502 and RG752 experimental groups. NMP at concentrations between 1 and 5 mM enhances BMP activity two to eight times and is most efficient at low BMP concentrations [9]. RG502, the 50 mol% lactide ISFI, may provide more optimal NMP concentrations for the greatest BMP enhancement due to its almost linear NMP release from the beginning up to 100% release on day 14 (Figure 2). During these 14 days, less than 20% of the BMP was released according to the *in vitro* experiment, but its activity might have been enhanced several fold by the released NMP. Subsequently, the remaining 80% of the BMP-2 was released between day 14 and 21 (Figure 6). Overall, one can speculate that in the ISFI group of 50 mol% lactide containing polymer, the defect was exposed to a more optimal BMP activity from the beginning by low amounts of rhBMP-2 enhanced by NMP and later by higher amounts of rhBMP-2 until day 21. In the 75 mol% lactide group, both NMP and BMP release were more sustained until day 28, making it less likely that NMP or BMP threshold levels were reached so that the overall BMP activity remained inadequate to significantly stimulate bone regeneration.

5. Conclusion

Taken together, our present findings demonstrate the possibility to use *in situ* forming implants for the dual delivery of rhBMP-2 and its enhancer NMP. For the rhBMP-2 to remain active, the growth factor has to be directly dissolved in aqueous

solution to replace up to 10% of the NMP content. Dependent on the polymer molecular weight and percentage of lactide of the ISFI system, release kinetics for NMP and rhBMP-2 can be tuned and sustained release over 28 days can be achieved. For bone regeneration purposes in a critical size defect in the calvarial bone of rabbits, the faster degrading and faster NMP and rhBMP-2 releasing system with 50 mol% lactide performed better in terms of bone bridging and significantly better for the amount of the bony regenerated area in the defect. Since the optimal release kinetics of NMP and rhBMP-2 might vary between applications, the ISFI systems have to be matched to the specific requirements.

Acknowledgements

We thank Mi Liu for help with GPC analysis and Yvonne Bloemhard and Alexander Tchouboukov for excellent technical assistance. This research work was partly supported by grants from the Swiss National Science Foundation (31003A 140868) and (CR32I3_152809) and by AOCMF (project C-10-37W), the Craniomaxillofacial Specialty of the AO Foundation.

References

[1] V. Rosen, BMP2 signaling in bone development and repair, Cytokine & Growth Factor Reviews, 20 (2009) 475-480.
[2] P.C. Bessa, M. Casal, R.L. Reis, Bone morphogenetic proteins in tissue engineering: the road from laboratory to clinic, part II (BMP delivery), Journal of tissue engineering and regenerative medicine, 2 (2008) 81-96.
[3] Z.S. Haidar, R.C. Hamdy, M. Tabrizian, Delivery of recombinant bone morphogenetic proteins for bone regeneration and repair. Part A: Current challenges in BMP delivery, Biotechnology Letters, 31 (2009) 1817-1824.

[4] Z.S. Haidar, R.C. Hamdy, M. Tabrizian, Delivery of recombinant bone morphogenetic proteins for bone regeneration and repair. Part B: Delivery systems for BMPs in orthopaedic and craniofacial tissue engineering, Biotechnology Letters, 31 (2009) 1825-1835.
[5] U. Ripamonti, A.H. Reddi, Tissue engineering, morphogenesis, and regeneration of the periodontal tissues by bone morphogenetic proteins, Critical Reviews in Oral Biology & Medicine, 8 (1997) 154-163.
[6] H. Seeherman, J.M. Wozney, Delivery of bone morphogenetic proteins for orthopedic tissue regeneration, Cytokine & Growth Factor Reviews, 16 (2005) 329-345.
[7] W. Zhu, J. Kim, C. Cheng, B.A. Rawlins, O. Boachie-Adjei, R.G. Crystal, C. Hidaka, Noggin regulation of bone morphogenetic protein (BMP) 2/7 heterodimer activity in vitro, Bone, 39 (2006) 61-71.
[8] E.J. Carragee, E.L. Hurwitz, B.K. Weiner, A critical review of recombinant human bone morphogenetic protein-2 trials in spinal surgery: emerging safety concerns and lessons learned, Spine Journal, 11 (2011) 471-491.
[9] B.S. Miguel, C. Ghayor, M. Ehrbar, R.E. Jung, R.A. Zwahlen, P. Hortschansky, H.G. Schmoekel, F.E. Weber, N-methyl pyrrolidone as a potent bone morphogenetic protein enhancer for bone tissue regeneration, Tissue Eng Part A, 15 (2009) 2955-2963.
[10] C. Ghayor, R.M. Correro, K. Lange, L.S. Karfeld-Sulzer, K.W. Graetz, F.E. Weber, Inhibition of Osteoclast Differentiation and Bone Resorption by N-Methylpyrrolidone, Journal of Biological Chemistry, 286 (2011) 24458-24466.
[11] K.P. Lee, N.C. Chromey, R. Culik, J.R. Barnes, P.W. Schneider, TOXICITY OF N-METHYL-2-PYRROLIDONE (NMP) - TERATOGENIC, SUBCHRONIC, AND 2-YEAR INHALATION STUDIES, Fundamental and Applied Toxicology, 9 (1987) 222-235.
[12] Food and Drug Administration, Guidance for Industry QC3 - Tables and List, in: U.S.H.a.H. Services (Ed.), Silver Spring, MD, 2012.
[13] E.B. Hunziker, L. Enggist, A. Kuffer, D. Buser, Y. Liu, Osseointegration: The slow delivery of BMP-2 enhances osteoinductivity, Bone, 51 (2012) 98-106.
[14] K. Sitarek, A. Kilanowicz, Tissue distribution and excretion of N-methyl-2-pyrrolidone in male and female rats, Int J Occup Med Environ Health, 19 (2006) 142-148.
[15] B. Akesson, B.A. Jonsson, Major metabolic pathway for N-methyl-2-pyrrolidone in humans, Drug Metab Dispos, 25 (1997) 267-269.
[16] A.J. McHugh, The role of polymer membrane formation in sustained release drug delivery systems, J Control Release, 109 (2005) 211-221.
[17] R.L. Dunn, The Atrigel Drug Delivery System, in: R.J. Rathbone, J. Hadgraft, M.S. Roberts (Eds.) Modified-Release Drug Delivery Technology, Marcel Dekker, New York, 2002.
[18] D. Kim, T. Kang, D. Gober, C. Orlich, A liquid membrane as a barrier membrane for guided bone regeneration, ISRN Dent, 2011 (2011) 468282.
[19] G.L. Southard, R.L. Dunn, S. Garrett, The drug delivery and biomaterial attributes of the ATRIGEL technology in the treatment of periodontal disease, Expert opinion on investigational drugs, 7 (1998) 1483-1491.
[20] W. Schloegl, V. Marschall, M.Y. Witting, E. Volkmer, I. Drosse, U. Leicht, M. Schieker, M. Wiggenhorn, F. Schaubhut, S. Zahler, W. Friess, Porosity and mechanically optimized PLGA based in situ hardening systems, Eur J Pharm Biopharm, 82 (2012) 554-562.
[21] G.L. Southard, R.L. Dunn, S. Garrett, The drug delivery and biomaterial attributes of the ATRIGEL technology in the treatment of periodontal disease, Expert Opin Investig Drugs, 7 (1998) 1483-1491.
[22] F.E. Weber, G. Eyrich, K.W. Gratz, R.M. Thomas, F.E. Maly, H.F. Sailer, Disulfide bridge conformers of mature BMP are inhibitors for heterotopic ossification, Biochemical and Biophysical Research Communications, 286 (2001) 554-558.
[23] M.R. Lutolf, F.E. Weber, H.G. Schmoekel, J.C. Schense, T. Kohler, R. Muller, J.A. Hubbell, Repair of bone defects using synthetic mimetics of collagenous extracellular matrices, Nature Biotechnology, 21 (2003) 513-518.

[24] M. de Wild, R. Schumacher, K. Mayer, E. Schkommodau, D. Thoma, M. Bredell, A. Kruse Gujer, K.W. Gratz, F.E. Weber, Bone Regeneration by the Osteoconductivity of Porous Titanium Implants Manufactured by Selective Laser Melting: A Histological and Micro Computed Tomography Study in the Rabbit, Tissue Eng Part A, 19 (2013) 2645-2654.
[25] A. Kruse, R.E. Jung, F. Nichols, R.A. Zwahlen, C.H.F. Hämmerle, K.W. Grätz, F.E. Weber, Bone regeneration in the presence of a synthetic hydroxyapatite oxide based and a xenogenic hydroxyapatite based bone substitute material, EAO Warschau, (2008).
[26] P.R. Schmidlin, A. Tchouboukov, F.J. Wegehaupt, F.E. Weber, Effect of cerium chloride application on fibroblast and osteoblast proliferation and differentiation, Arch Oral Biol, 57 (2012) 892-897.
[27] H. Liu, S.S. Venkatraman, Effect of polymer type on the dynamics of phase inversion and drug release in injectable in situ gelling systems, J Biomater Sci Polym Ed, 23 (2012) 251-266.
[28] H.K. Makadia, S.J. Siegel, Poly Lactic-co-Glycolic Acid (PLGA) as Biodegradable Controlled Drug Delivery Carrier, Polymers (Basel), 3 (2011) 1377-1397.
[29] L. Solorio, A.M. Olear, J.I. Hamilton, R.B. Patel, A.C. Beiswenger, J.E. Wallace, H. Zhou, A.A. Exner, Noninvasive characterization of the effect of varying PLGA molecular weight blends on in situ forming implant behavior using ultrasound imaging, Theranostics, 2 (2012) 1064-1077.
[30] K.J. Brodbeck, J.R. DesNoyer, A.J. McHugh, Phase inversion dynamics of PLGA solutions related to drug delivery. Part II. The role of solution thermodynamics and bath-side mass transfer, J Control Release, 62 (1999) 333-344.
[31] R.B. Patel, A.N. Carlson, L. Solorio, A.A. Exner, Characterization of formulation parameters affecting low molecular weight drug release from in situ forming drug delivery systems, J Biomed Mater Res A, 94 (2010) 476-484.
[32] R.B. Patel, L. Solorio, H. Wu, T. Krupka, A.A. Exner, Effect of injection site on in situ implant formation and drug release in vivo, J Control Release, 147 (2010) 350-358.
[33] P.D. Graham, K.J. Brodbeck, A.J. McHugh, Phase inversion dynamics of PLGA solutions related to drug delivery, J Control Release, 58 (1999) 233-245.
[34] X. Luan, R. Bodmeier, Influence of the poly(lactide-co-glycolide) type on the leuprolide release from in situ forming microparticle systems, J Control Release, 110 (2006) 266-272.
[35] G. Bogle, S. Garrett, R. Dunn, Polymer delivery systems and BMP's in periodontal regeneration: Pilot studies, Journal of Dental Research, 77 (1998) 196-196.

Figure legends:

Figure 1: NMP release dependence on molecular weight. Weight average of RG502 is 14.5 kDa and of RG503 42.6 kDa. Cumulative NMP release of ISFI with 40% PLGA and 50 mol% lactide. Release over (a) 24 h (b) and 28 d. Mean ± SD (n=3).

Figure 2: NMP release dependence on lactide percentage. Lactide content for RG502 amounts to 50 mol%, of RG752 to 75 mol% and of R202 to 100 mol%. Cumulative NMP release of ISFI with 40% PLGA and the lowest molecular weight. Release over (a) 24 h (b) and 28 d. Mean ± SD (n=3).

Figure 3: Degradation of ISFI prepared with RG502 or RG503 in PBS. Mean ± SD (n=3).

Figure 4: Activity of BMP-2 with NMP. Alkaline phosphatase activity was measured with lyophilized BMP-2 directly diluted in various percentage (w/w) NMP solutions with acetic acid (black bars) or BMP-2 first dissolved in an aqueous acetic acid solution and then diluted in various percentage NMP solutions (gray diagonal hashed bars). Cells only and NMP served as negative controls and BMP-2 (in aqueous solution) and BMP-2 (in aqueous solution) and NMP added directly to the well served as positive controls (solid gray bars). All data is normalized to the positive control of BMP-2 (in aqueous solution). Mean ± SD (n=3).

Figure 5: NMP release dependence on percentage of water in the solvent. Cumulative NMP release of ISFI with 40% PLGA, 50 mol% lactide, and the lowest molecular weight (RG502). The solvent dissolving the polymers was entirely NMP (no water), contained 8.3 or 10% (w/w) water. No BMP-2 is included in these formulations. Release over (a) 24 h (b) and 28 days. Mean ± SD (n=3).

Figure 6: BMP-2 release dependence on lactide percentage. BMP-2 was included in the ISFI solutions and released into a PBS-urea buffer. BMP-2 was measured with a Bio-Dot apparatus with a primary anti-BMP-2/4 antibody. All data are normalized to the control of rhBMP-2 in PBS-urea at the same timepoint. Mean ± SD (n=3).

Figure 7: In vivo bone formation. Titanium based lattice scaffolds (17/15 mm) in diameter were manufactured by selective laser melting, filled with ISFI and implanted in the calvarial bone of rabbits. a) Design of the scaffold. b) Loading of the scaffold with ISFI. c) Defect before implant placement. d) Placement of scaffold into the bony defect. Histology of the middle section: e) control is scaffold only, f) scaffold with ISFI 50% mol% lactide without BMP, g) scaffold with ISFI 75 mol% lactide with BMP, h) scaffold with ISFI 50 mol% lactide with BMP, scale in mm is provided in e; bone appears blue with Toluidine-blue staining, titanium black. i) Box–plot for defect bridging and k) bony regenerated area. * indicates P values<0.05. Exact P values are provided in the result section. Mean ± SD (n=6).